

Supporting Information

Synthesis of Aminoacylated tRNA

The pdCpA dinucleotide (tetrabutylammonium salt) and a sample of α OH-Phenylalanyl-dCA were obtained as a gift from Neurion Pharmaceuticals (Pasadena, CA). Subsequent preparation of α OH-Phenylalanyl-dCA was carried out according to the protocols in references 17-18. An example synthesis is described below.

Synthesis of α OH-Phenylalanine cyanomethyl ester (1)

L-phenylactic acid (266 mg, 1.6 mmol) was dissolved in 3 mL DMF. To this was added chloroacetonitrile (3 mL, 47.4 mmol) and TEA (651 μ L, 4.6 mmol). The reaction was allowed to proceed under nitrogen at room temperature overnight. The desired product was purified by flash chromatography (silica gel, 3:7 EtOAc:Hexanes). The final yield (amber oil) was 18.9 mg (68%). Analysis by ESI-MS (Expected $[M+Na]^+=228.07$, Observed $[M+Na]^+=227.8$)

Synthesis of α OH-Phenylalanine-dCA (2)

α OH-Phenylalanine cyanomethyl ester (11 mg, 54 μ mol) was dissolved in 400 μ L DMF and added to the tetrabutyl ammonium salt of pdCpA (9 μ mol) in the presence of a catalytic amount of TBA-acetate. The reaction was allowed to proceed under nitrogen for 4 hr. at room temperature. The aminoacylated dinucleotide was purified by RP-HPLC using a gradient from 25 mM NH_4OAc (pH=4.5) to CH_3CN . Following lyophilization of the pooled fractions, the product was dissolved in 10 mM HOAc and lyophilized again. The final yield was determined by absorbance at 260 nm. and found to be 97 nmol (1%). The product was analyzed by ESI-MS (Expected $[M-H]^- = 783.16$, Observed $[M-H]^- = 783.2$).

In vitro Transcription of THG73 tRNA

The plasmid harboring the THG73 gene was linearized with FokI and transcribed with T7 RNA polymerase. The transcription product was gel-purified by Urea-PAGE, dissolved in dH_2O , and quantitated by absorbance at 260 nm.

Ligation to THG73 tRNA

20 μ g THG73 tRNA (8 μ L in dH_2O) in HEPES (22 μ L, 10 mM, pH=7.5) was heated to 94 $^{\circ}C$ for 3 min and allowed to cool slowly at room temperature. 8 μ L α OH-Phenylalanine-dCA (3 mM in DMSO), 32 μ L 2.5 X Reaction Buffer (25 μ L 400 mM HEPES pH=7.5, 10 μ L 100 mM DTT, 25 μ L 200 mM $MgCl_2$, 3.75 μ L 10 mM ATP, 10 μ L

5 mg/mL BSA, 26.25 μ L dH₂O), 7 μ L water, and 4 μ L T4 RNA Ligase (N.E.B.). After incubation at 37 °C for 1 hr, the reaction was extracted once with an equal volume of phenol (saturated with 300 mM NaOAc, pH=5.0:CHCl₃:isoamyl alcohol (25:24:1)) and ethanol precipitated. The pellet was washed with 70% EtOH, dried under vacuum, and dissolved in 1 mM NaOAc to a concentration of 2 μ g/ μ L.

Construction of Fusion Templates (Phe(K), UAG(K), UAG(V))

These dsDNA templates were created by PCR using overlapping primers: (Forward primer = **Gen-FP**: 5'-TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA-3') and a unique reverse primer (**Phe(K)-RP**: 5'-CTTAACAACGAAAACAACCATTGTAATTGTAAATAGTAATTG-3', **UAG(K)-RP**: 5'-CTTAACAACCTAAACAACCATTGTAATTGTAAATAGTAATTG-3', **UAG(V)-RP**: 5'...AACAAACCTAAACAACCATTGTAATTGTAAATAGTAATTG...3'). Prior to PCR, all primers were purified by Urea-PAGE. Following PCR, the dsDNA templates were transcribed into mRNA by T7 runoff transcription and purified by Urea-PAGE. The mRNA was ligated to F30P (5'-dA₂₁[C₉]₃dAdCdC-P; C₉=triethylene glycol phosphate (Glen Research), P= puromycin (Glen Research)), a flexible DNA linker containing puromycin. 5' phosphorylated F30P was ligated to the mRNA in the presence of an oligonucleotide splint (**Phe(K)-splint**: 5'-TTTTTTTTTTTTTCTTAACAACGAAA-3', **UAG(K)-splint**: 5'-TTTTTTTTTTTTTCTTAACAACCTAA-3', **UAG(V)-splint**: 5'...TTTTTTTTTTTTTAACAACAACCTAA...3'). The ratio of RNA:PF30P:splint was 1:1.2:1.4. 4 μ L of T4 DNA Ligase (N.E.B.) was added and the reaction was carried out at room temperature for 1 hr. The mRNA-DNA template was gel purified by Urea-PAGE, dissolved in water, and quantitated by absorbance at 260 nm.

Construction of MK(n) Library Templates (MK0, MK2, MK4, MK6, MK8, MK10)

Antisense ssDNA templates were synthesized at the Keck Oligonucleotide Synthesis Facility (Yale). The sequences for the MK Library ssDNA templates are as follows:

MK(2): 5'-TCCGCTGCCGGATTCTASNNNSNNCATTGTAATTGTAAATAGTAATTG-3'
MK(4): 5'-TCCGCTGCCGGATTCTASNNNSNNNSNNCATTGTAATTGTAAATAGTAATTG-3'
MK(6): 5'-TCCGCTGCCGGATTCTASNNNSNNNSNNNSNNCATTGTAATTGTAAATAGTAATTG-3'
MK(8): 5'-TCCGCTGCCGGATTCTASNNNSNNNSNNNSNNNSNNCATTGTAATTGTAAATAGTAATTG-3'
MK(10): 5'-TCCGCTGCCGGATTCTASNNNSNNNSNNNSNNNSNNNSNNCATTGTAATTGTAAATAGTAATTG-3'

N=A,T,C,G S=G,C

The reagent bottle used for the “N” positions was made by mixing A:C:G:T in the ratio 3:3:2:2. The reagent bottle

for “S” positions was made by mixing C:G in a 3:2 ratio.

MK library dsDNA was amplified by PCR using the forward primer Gen-FP (5'-TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA-3') and the reverse primer MKLib-RP (5'-GCCAGATCCGCTGCCGGATTTCTA-3'). The mRNA was made by *in vitro* T7 runoff transcription as described above. Following PAGE purification, the mRNA was ligated to F30P via. an oligonucleotide splint (**MKLib-splint**: 5'-TTTTTTTTTTTTTGCCAGATCCGCTG-3'). Following PAGE purification of the ligation reaction, the templates were dissolved in water and quantitated by absorbance at 260 nm.

Translation of mRNA-peptide fusions

Templates were translated in rabbit reticulocyte lysate under standard conditions with a final template concentration of 400 nM. For templates with UAG amber codons (UAG(K), UAG(V), and the MK Libraries), the reaction mixture was supplemented with 2 µg αOH-phenylalanyl-tRNA/25 µL translation volume. ³⁵S-Methionine was used as the labeling agent. After 1 hr of translation at 30 °C, KOAc and MgCl₂ were added to a final concentration of 600 mM and 50 mM respectively, and the reactions were placed at -20 °C overnight.

Fusion Purification

The translation mixture was diluted 1:10 in dT Binding Buffer (10 mg/mL dT cellulose, 1M NaCl, 20 mM Tris, 1 mM EDTA, 0.2% Triton X-100, pH=8) and agitated for 1 hr. at 4 °C. The dT cellulose was filtered and washed with dT Wash Buffer (300 mM NaCl, 20 mM Tris, pH=8). The DNA-peptide conjugates were eluted with 10 mM NH₄OH and ethanol precipitated in the presence of linear acrylamide (Ambion).

Reaction with DSG and Determination of Cyclization Efficiency by NH₄OH Hydrolysis

Purified fusions (dissolved in water) were diluted 1:1 in 100 mM phosphate buffer (pH=8). To this was added 1 mg/mL DSG (in DMF) to a final concentration of 600 µM. The final concentration of fusion in these reactions was estimated to be 1-10 nM by scintillation counting of the ³⁵S radiolabel. The reaction was allowed to proceed for 1 hr at room temperature, treated with RNase Cocktail (Ambion) at room temperature for 2 hr, and diluted 1:1 in 2M NH₄OH and heated at 65 °C for 30 min to hydrolyze the internal ester linkage. The fusions were treated with RNase to facilitate resolution of the reaction products by Urea-PAGE. The sample was dried *in vacuo*, co-evaporated with water, and redissolved in Urea-PAGE loading buffer (80% formamide, bromophenol blue). The

reaction products were separated on a 15% Urea-PAGE gel at 15W for 90 min. The gel was fixed, dried, and visualized by autoradiography. The fraction cyclized was determined by subtracting the band volume of the mock-reacted sample (-DSG/+NH₄OH) from that of the DSG reacted sample (+DSG/+NH₄OH) and dividing the result by the band volume of the control sample (+DSG/-NH₄OH).

The Urea-PAGE gel shown below (Figure 1s) is representative of a typical experiment to determine the cyclization efficiencies of the MK libraries (MK2-MK10). The major band represents the full-length desired fusion product and is dependent on the addition of aminoacylated suppressor tRNA. The high and low mobility bands are observed in both the presence and absence of supplemented aminoacyl-tRNA and are not affected by base treatment. The high mobility band is most likely due to internal initiation of translation while the low mobility band may arise from translational frameshifting at the UAG stop codon.

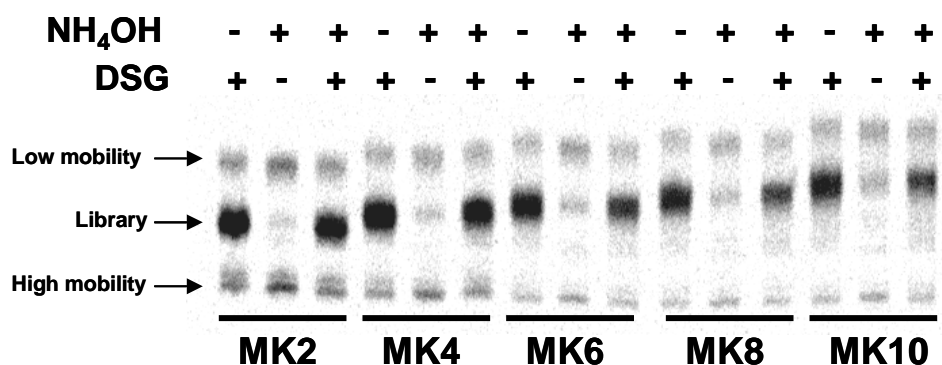


Figure 1s: Urea-PAGE of MK Library hydrolysis products. Quantitation of cyclization efficiency is described above.

Analysis of Fusions by MALDI-TOF MS.

200 pmol of Phe(K) Template was translated in the absence of ³⁵S methionine as described above. The translation reactions were dT-purified, ethanol precipitated, and dissolved in 50 mM phosphate buffer (pH=8). These were treated with either DMF (control) or DSG for 1 hr at room temperature. The RNA was then removed by incubation with RNase cocktail (Ambion) at room temperature for 3 hr. Previous experiments showed that removal of the RNA was essential for observation of the mass signal in MALDI-TOF MS. The peptide-DNA fusions were then dT-purified, eluted with 10 mM NH₄OH, and quenched with 10% HOAc. The samples were concentrated and desalted with a Nanosep 3K centrifugal filtration device (Pall). The final desalted sample was brought to a volume of 10 µL by evaporation. A portion of this sample was incubated for 30 seconds with DOWEX ion exchange resin (previously treated with NH₄OAc).¹ Approximately 0.15 µL was applied to the matrix spot (3-hydroxypicolinic

acid) and allowed to dry. This was repeated 9 times to increase sample concentration. Methionine oxidation was accomplished by treating the dried sample with 0.25 μL of 0.5% H_2O_2 prior to analysis.² This was found to quantitatively oxidize the methionine side chain thioether to the sulfoxide form, effectively simplifying the spectra (data not shown).

All MALDI were obtained on an Applied Biosystems Voyager DE PRO MALDI-TOF mass spectrometer operating in linear positive ion mode with delayed extraction. The accelerating voltage was set at 25000 V, grid voltage at 92.5%, and guide wire at 0.15%. The extraction delay time was 325 nsec. A five point external protein calibration (matrix: sinapic acid) was used. The laser power was adjusted to produce the strongest mass signal for the peptide-DNA conjugates. A table of predicted and observed masses for the linear and DSG-reacted fusions as well as the F30P linker is shown below:

Species	Predicted [M+H]⁺	Observed [M+H]⁺
F30P	8573.72	8582.2
F30P(mono-acylated)	8687.75	8697.27
Phe(K)-F30P(linear)	9392.19	9401.08
Phe(K)-F30P(cyclic)	9488.21	9497.75

Table 1s: Predicted and Observed [M+H]⁺ values for fusion species described in this experiment. Predicted values represent exact mass calculations (ChemDraw).

Alternate Cyclization Products

The construction of the libraries described in this manuscript allows the incorporation of lysine in the random region via the AAG codon. The chance of lysine insertion at any of the randomized positions is therefore approximately 1/32. Thus, for the n = 2, 4, 6, 8, and 10 libraries, the fraction of sequences in the library containing one or more internal lysines is 0.062, 0.12, 0.19, 0.25, and 0.31 respectively. Thus, between ~94% (n=2) and ~70% (n=10) of the library members have the potential to form only the desired macrocyclic product.

The majority of the remaining library members will have one internal lysine encoded in the randomized cassette region. Library members with one internal lysine have the potential to form 3 types of cyclization products: 1) the intended cyclization product (N-terminus-fixed lysine), 2) fixed lysine-cassette lysine, and 3) cassette lysine-N-terminus. For these molecules, one template sequence corresponds to three different cyclic products, providing

the ability to select functional molecules from each category. After a selection is completed, chemical synthesis can be used to construct each distinct cyclic product and thereby determine which is functional.

NHS Reactivity with mRNA-peptide Fusions

Figure 2s shows that biotin-NHS reacts nearly quantitatively with the Phe(K) fusions. Reaction with different bis-NHS cross-linkers prior to reaction with biotin-NHS greatly reduces fusion binding to neutravidin agarose, implying that all NHS cross-linkers used here may be used to cyclize fusions.

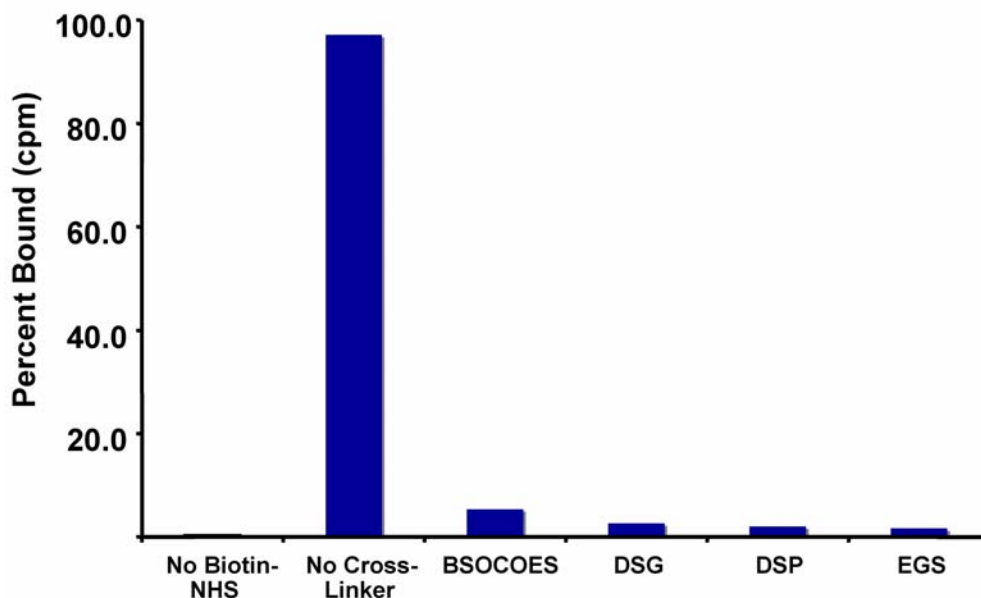


Figure 2s: Biotin-NHS reactivity interference using different NHS-based cross-linkers. dT-purified Phe(K) fusions were first reacted with 2.5 mM cross-linkers (BSOCOES = Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, DSG = disuccinimidyl glutarate, DSP = dithiobis[succinimidylpropionate], and EGS = ethylene glycol bis[succinimidylsuccinate]; all cross-linkers were from Pierce Chemical) or with DMSO only (No biotin-NHS and No Cross-linker) in 100 mM phosphate buffer (pH = 7.5), 10% (v/v) DMSO for 3 hours at room temperature. The reactions were then dT purified, ethanol precipitated, and reacted with biotin-NHS or with DMSO only (No Biotin-NHS) for 2 hours at room temperature. The products were again dT purified, eluted in H₂O, and incubated with 50 μ L of a 50/50 (v/v) slurry of Neutravidin agarose in 1X PBST (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4, 0.1 % (v/v) Triton X-100) for one hour at 4 °C. The agarose was washed with 1X PBST and the beads counted on a scintillation counter.

Supplemental Information (References):

- 1) Petersson, E.J.Shahgholi, M., Lester, H.A., Dougherty, D.A. *RNA* **2002**, 8, 542-547.
- 2) Corless, S., Cramer, R. *Rapid. Comm. Mass. Spec.* **2003**, 17(11), 1212-1215.